Critical Reviews™ in Oncogenesis, 7(5&6):433–456 (1996)

Tolerance and Cancer: A Critical Issue in Tumor Immunology

*Eduardo M. Sotomayor, Ivan Borrello, and Hyam I. Levitsky**

Department of Oncology, Johns Hopkins University School of Medicine, 720 Rutland Avenue/ 347 Ross Building, Baltimore, MD 21205–2196

* Author to whom all correspondence should be addressed.

ABSTRACT: Self-antigens are the most relevant and abundant antigens to which the host's immune system must be tolerant. Induction and maintenance of tolerance to self-antigens is mediated by several mechanisms that prevent inappropriate damage to normal tissues. However, these same mechanisms may impose potential barriers for the full development of effective immune responses against antigens expressed by tumors. A critical issue in tumor immunology is whether antigen presented by a progressively expanding tumor cell population results in T-cell tolerance. Utilizing a T cell receptor transgenic mice specific for a model tumor antigen expressed on a B-cell lymphoma, recently we have obtained direct evidence supporting the existence of tumor-induced antigen-specific tolerance. A better identification and understanding of the factor(s) involved in tumor-induced tolerance has clear implications for the development of novel cancer immunotherapies aimed at safely breaking tolerance, for example, releasing the brakes on antitumor immune responses while still limiting the induction of undesirable autoimmune responses.

KEY WORDS: anergy, antigen-specific tolerance, clonal deletion, clonal diversion, immunosuppression.

I. INTRODUCTION

For many years, tumor immunologists have debated how is it that tumors arise in immunocompetent host and avoid immunologic rejection, ultimately progressing to widely disseminated cancer. Initially, it was thought that because cancer cells are the transformed counterparts of normal cells and tissues they might simply fail to express proteins that can serve as tumor-rejection antigens. This rationale, as well as disappointing results with early immuno-therapeutic approaches, i.e., Bacille Calmette-Guerin (BCG), resulted in an overall negative impression and lack of optimism toward the field of tumor immunology. However, in recent years this field has undergone an impressive revival due to the identification of tumor antigens recognized by T cells as well as a more complete understanding of factors regulating immune responses (Boon and van der Bruggen, 1996; Pardoll, 1993). Several novel strategies have been designed by different groups trying to enhance the immune responses against tumors, both in experimental models as well as in clinical trials (Towsend and Allison, 1993; Dranoff et al., 1993; Hsu et al., 1996; Gong et al., 1997; Simons et al., 1997). Despite the initial optimism generated by these new immunotherapeutic tools, tumor immunologists faced the problem that, while these strategies often result in eradication of small tumor burden, they almost always fail when the tumor is more advanced or has been present for longer periods of time. While the exact explanation for this observation is not entirely clear, it pointed out that tumor progression may affect the capacity of the immune system to function properly and to respond in a positive way to immune-enhancing therapeutic approaches. One mechanism that has gained particular attention in recent years is the *induction of tolerance* to tumor antigens. As presented in this review, *tolerance* to self-antigens represent a normal mechanism to prevent inappropriate damage to normal tissues. However, these same mechanisms may blunt the ability of the immune system

0893-9675/96/$5.00

to recognize and respond to antigens expressed by tumors. In the clinical arena, it should be pointed out that at the time that a patient has the diagnosis of cancer, that patient's immune system most likely has been exposed to their tumor antigens probably for years while still allowing the development of the tumor (Pardoll, 1996). At this point, the immune system of the patient may well have been rendered tolerant to the tumor. A critical question in tumor immunology is whether antigen presented by a progressively expanding tumor cell population results in T-cell tolerance. Utilizing a T-cell receptor transgenic mice specific for a model tumor antigen expressed on a B-cell lymphoma, recently we have obtained direct evidence supporting the existence of tumor-induced antigen-specific tolerance that develops with tumor progression. Current studies are focusing on the mechanism responsible for the initiation and maintenance of tolerance to tumor antigens, and, even more importantly, whether this state of unresponsiveness can be safely reverted. In this avenue, the recent knowledge acquired in antigen processing and presentation as well as T-cell activation and regulation are leading to the generation of more potent immune-therapeutic tools capable of breaking tolerance and the long-elusive success of active immunotherapy.

II. BACKGROUND

A half a century ago, R. D. Owen and his colleagues at the University of Wisconsin, Madison, made a remarkable observation that would set up the basis of what we now know as *immunologic tolerance*. These investigators found that cattle dizygotic twins possessed not only their own red blood cells but also red cells that were derived from their twin partners, representing a natural example of cellular chimeras (Owen, 1945). F. R. Lillie had previously reported that cattle dizygotic twins are synchoral, that is, they share the same placenta and therefore their circulatory systems (Lillie, 1916). Owen and colleagues correctly postulated that because of this free exchange of blood during fetal development—involving not only blood cells, but also precursors or stem cells—a state of hematopoietic chimerism was generated that allowed the presence in each adult twin of both their own as well as its sibling's blood cells.

The immunologic implications of Owen's fascinating observation for immune tolerance was highlighted a few years later when Burnet and Fenner published the second edition of *The Production of Antibodies* (Burnet and Fenner, 1949) in which they formulated "the self-marker hypothesis" to explain why the phenomenon of autoimmunity is relatively rare rather than frequent in normal individuals (reviewed by Brent, 1997). Burnet and Fenner postulated that during embryonic development or early life the immune system undergoes a critical period learning how to tolerate self-markers while retaining the ability to recognize and react against foreign antigens. Taking the mosaic cattle dizygotic twins as an example, they suggested that the immune system of each twin became tolerant to any blood cell antigen of its sibling's that was present during their fetal development. Moreover, Burnet and Fenner postulated that the potential mechanism involved in the induction of this tolerance was related to the physical elimination (*clonal deletion theory*) of the lymphocytes that recognize and react with the sibling's blood cell antigens.

In the 1950s Billingham, Brent, and Medawar (Billingham et al., 1953; Billingham et al., 1956), working in London, provided the experimental support to Burnet's theory of self-tolerance induction. These investigators demonstrated that while adult mice reject skin grafts from immunologically foreign animals, tolerance to these allografts could be induced when viable cells from the allogeneic donor were injected into fetal and newborn mice. From these pivotal studies, important conclusions were obtained:

1. Induction of tolerance was seen when allogeneic cells were injected into experimental animals either during fetal development or in the early neonatal period. Later in life, any attempt to induce tolerance using this experimental approach was unsuccessful.
2. Tolerance was highly specific, and it was accompanied by the presence of donor cells in the tissues of the tolerant host.
3. Different degrees of tolerance were observed, from permanent to transient acceptance of skin grafts.

4. It was postulated that during fetal development the immune cells of the experimental animal learn to accept the foreign cells as "self" with the subsequent induction of tolerance similar to what occurs with the animal's own cells.

It should be pointed out that although the studies of Medawar and colleagues elegantly showed that tolerance to antigens can be induced during development of the immune system, they did not prove that *clonal deletion*—as proposed by Burnet—was the mechanism involved in the induction of the observed unresponsiveness.

In later years the phenomenon of immunologic tolerance was demonstrated in several species, in different tissues and organs, and for a variety of antigens. Therefore, ***immunologic tolerance***—defined as a state of unresponsiveness to a particular antigen—became relevant not only in the context of tissue transplantation but also to understanding normal development. In fact, elucidating the cellular and molecular mechanisms mediating immunologic tolerance has become central to understanding the "*self from non-self recognition*" theory that is considered by many to be the most important characteristic of the immune system.

Self-antigens represent the most relevant and abundant antigens to which the host's immune system must be tolerant. Therefore, the induction and maintenance of this unresponsiveness is critical in normal individuals, because disruption of this tolerant state to self-antigens has been shown to result in overt autoimmune responses. However, as discussed in this review, the same mechanism(s) that normally prevent inappropriate attack against self-antigens may impose potential barriers for the development of effective immune responses against antigens expressed by tumors. Several groups, including our own, are currently focusing on the study of the mechanisms involved in the induction and maintenance of tolerance to tumor antigens and, even more relevantly, whether this state can be reverted. A better understanding of the factor(s) involved in tumor-induced tolerance might therefore provide the basis for the generation of new immunotherapeutic tools capable of releasing the brakes on antitumor immune responses while still limiting the induction of undesirable autoimmune responses.

III. CATEGORIES OF TOLERANCE

A. Central Tolerance

The ability of the immune system to specifically recognize a vast array of foreign antigens is achieved by the random generation of antigen receptors on B and T cells having diverse specificities. The genetic basis for this diversity of antigen recognition is the ordered rearrangement of immunoglobulin or T-cell receptor genes during lymphocyte development. For T cells, this process occurs in the thymus. Because the generation of T-cell receptors is random, a selection process exists to eliminate T cells whose receptors either fail to recognize any antigen presented by host MHC molecules or those having too high an affinity for self-antigen. This process is known as *positive* and *negative* selection, respectively.

1. Negative Selection

A large body of evidence has been accumulated indicating that negative selection through clonal deletion of autoreactive T cells represents the most important mechanism of central tolerance. In very elegant studies, Kappler and Marrack provided the experimental support for the clonal deletion model of self-tolerance. These investigators took advantage of the availability of monoclonal antibodies (KJ23) that react with all αβ T-cell receptors that expressed the product of a particular β variable region gene-denominated Vβ17. They found that almost all T cells whose TCR β component utilized Vβ17 reacted with cells expressing the murine MHC class II molecule I-E. This system allowed these investigators to follow the fate of T cells and T-cell precursors of known reactivity against MHC class II molecule I-E. They found that in mouse strains that do not express I-E molecules—because their germline I-E α gene is defective—the Vβ17 product was expressed in 5 to 10% of their T cell repertoire. In sharp contrast, in the C57BR mouse strain—in which I-E$^+$ is self-antigen—there were

almost no T cells that expressed the Vβ17 product. Analysis of the thymocytes of I-E$^+$ mice revealed that potentially self-reacting T cells expressing Vβ17 were present in the immature CD4$^+$8$^+$ thymocyte population but were *selectively eliminated* from both the mature thymocyte pool as well as the peripheral T cell population in mice expressing I-E (Kappler et al., 1987). It was further demonstrated that full induction of negative selection requires intrathymic contact of T cells with the bone marrow-derived elements of the thymus (Marrack et al., 1988). Thus, these studies showed beyond reasonable doubt that a major mechanism for self-tolerance in developing T cells involves the physical elimination or "clonal deletion" of thymocytes expressing self-reactive TCRs and that this process occurs at an immature CD4$^+$8$^+$ stage (reviewed in Fowlkes and Pardoll, 1989; Nossal, 1994).

2. Positive Selection

This process favors the development of thymocytes expressing TCR's that are capable of recognizing peptides presented by self-MHC molecules. Von Boehmer and colleagues performed elegant experiments that illustrated the process of positive and negative selection in the thymus. (Kisielow et al., 1988; Scott et al., 1989; reviewed in von Boehmer, 1994). These investigators immunized a female H-2^b strain mouse with male cells from the same strain. Then T cells isolated from the female were repetitively stimulated *in vitro* with male spleen cells in order to generate a set of T cell clones that could specifically recognize the H-Y antigen (encoded by the male Y chromosome) plus H-2^b. A CD8$^+$ T cell clone was further characterized and found to recognize H-Y plus MHC class I H-2D^b. These investigators went one step further and cloned the rearranged α and β TCR from this CD8$^+$ T cell clone and generated transgenic mouse lines. These were crossed to two different background strains. The first was the strain of origin of the original T cell clone (H-2^b) and the other was a strain with a different MHC allele (H-2^d). Using a monoclonal antibody specific for the transgenic TCR, von Boehmer and colleagues could follow the developmental fate of T cells expressing that particular TCR. In the MHC incompatible H-2^d mice, thymocytes developed only to the CD4$^+$8$^+$ stage and then stopped without further differentiation. In the H-2^b mice, different patterns were seen in male mice vs. female mice. In male H-2^b mice, in which H-Y is a self-antigen, thymocytes expressing the transgenic TCR were deleted at the CD4$^+$8$^+$ stage due to *negative selection*. However, in the female H-2^b mice, in which H-Y is not expressed, thymocytes expressing the transgenic TCR developed beyond the CD4$^+$ 8$^+$ stage and all become CD4$^-$8$^+$. These cells were therefore *positively selected*. These experiments clearly showed that the developmental fate of a thymocyte depends on the fit between its T cell receptor and the MHC alleles and self-antigens expressed in the thymic microenvironment. Too good a fit and the T cells are deleted by negative selection; too poor a fit and the cells fail to be positively selected.

B. Peripheral Tolerance

Peripheral tolerance refers to the induction of unresponsiveness in mature lymphocytes after they have completed their development. As noted above, central tolerance provides a highly efficient mechanism for the physical elimination of T cells that have high affinity for antigen that is present in the thymus during T cell development. However, this still leaves open the question as to how the T cell arm of the immune system is rendered tolerant to tissue-specific self proteins uniquely expressed extrathymically or induced during organ development, cell death, or viral infection. To accomplish this task, the immune system has developed a number of mechanisms that carefully regulate T cell activation, and in most instances preclude attack against peripheral self-antigens (Tivol et al., 1995; van Parijs et al., 1996). In contrast to central tolerance, the mechanisms that mediate peripheral tolerance are much less well understood. The induction of an unresponsive state termed **anergy** rather than clonal deletion appears to be one of the most important mechanisms for tolerance in mature lymphocytes (Rammensee, 1989; Kawabe and Ochi, 1990; Schönrich, 1991). The advent of T cell receptor transgenic mice has provided an exceptional tool for studies in this area over the last several years.

This has resulted in the description of several different mechanisms that are involved in the induction and maintenance of peripheral tolerance. However, these mechanisms, operative in normal conditions to safeguard against the disastrous consequences of unregulated T cell activation, may impose significant barriers to the full development of effective antitumor responses against antigens expressed by tumors. This concern is particularly relevant given the recent discovery that certain tumor antigens represent lineage-specific tissue differentiation antigens also expressed in normal tissues rather than unique neoantigens solely expressed by tumor cells (Houghton, 1994; Boon and van der Bruggen, 1996; Robbins and Kawakami, 1996).

Therefore, in this review we focus initially on the classic experiments and systems that allowed the description of the different mechanisms of peripheral tolerance to normal self-antigens. This background will provide insight into the factor(s) that may be operative in the induction of T cell tolerance by tumors. Furthermore, the identification and understanding of these mechanism(s) has clear implications for the development of novel cancer immunotherapies aimed to the restoration of T cell function, as these are the very cells that must be recruited, activated, and amplified for any meaningful antitumor response to be generated.

1. Two Signal Model of Lymphocyte Activation

One of the most influential concepts that is essential to many of the models of peripheral tolerance is the so-called "*two signal model of lymphocyte activation*", originally proposed by Bretscher and Cohn (Bretscher and Cohn, 1970) and expanded upon in 1975 by Lafferty and Cunningham to account for the regulated response of lymphocytes after encountering antigen on antigen-presenting cells (Lafferty and Cunningham, 1975). Although these models were originally formulated to explain the regulated production of antibodies by B cells, more recently immunologists have applied this model to the regulation of T cell activation. In its simplest form the model states that at least **two signals** are required for appropriate T cell activation; **signal 1** is the signal delivered by the antigen-specific T cell receptor after encountering its peptide/MHC ligand on antigen-presenting cells, whereas **signal 2** represents a nonpolymorphic signal delivered by the APC to the T cell. The model predicts that T cell activation only occurs when T cells receive both signals from the APC. If only one of the two required signals is provided, that is, engagement of their antigen receptor without the second signal, T cells would be turned off rather than activated.

The two signal model of lymphocyte activation was further highlighted in seminal studies by Jenkins and Schwartz, who analyzed the ability of $CD4^+$ T cell clones propagated in culture to be stimulated by antigen-presenting cells (APCs) pulsed with the appropriate peptide antigen. In these experiments the read-out was IL-2 production by the T cell clone. It was discovered that if the APCs were chemically fixed prior to being mixed with peptide antigen plus the T cell clone, the T cells failed to make IL-2 (Jenkins and Schwartz, 1987a). This impairment in IL-2 production by T cell clones was also observed when the antigen was presented by purified class II MHC molecules in planar lipid membranes (Quill and Schwartz, 1987). However, more importantly, when the T cells that had encountered fixed APCs plus antigen were subsequently stimulated with freshly isolated APCs plus antigen (that normally resulted in T cell activation and IL-2 production), there was still no IL-2 production (Jenkins et al., 1987b). These results suggested that the T cells had somehow been rendered nonfunctional or "anergic" by their initial encounter with fixed APCs plus antigen. It has been shown subsequently that fixation destroys the ability of the APCs to deliver the second signal. The induction of this anergic state was *antigen dependent* (i.e., it required signal one), because T cells exposed to fixed APCs in the absence of antigen did not become unresponsive to a second stimulation with fresh APCs plus antigen. Consequently, the two-signal model was extended to not only require the two signals for appropriate T cell activation, but also to describe a form of *antigen-specific tolerance* that occurs as a consequence of receiving signal one (T cell receptor engagement by the APCs' MHC/peptide antigen complex) in the absence of signal two.

Subsequently, it was shown that T cell clonal anergy can be reverted when these clones were stimulated *in vitro* with IL-2 (Beverly et al., 1992) and that prevention of T cell anergy can be achieved by signaling through the γc chain of the IL-2 receptor (Boussiotis et al., 1994). The molecular basis for the profound block in IL-2 production has received significant attention in recent years. Kang et al. have shown that this block occurs at the level of transcription of the IL-2 gene and to involve a failure of a transcription factor, **activator protein-1** (AP)-1 (Kang et al., 1992). Examination of the signal transduction pathways in anergic T cell clones revealed an altered protein thyrosine phosphorylation pattern involving $p56^{lck}$ and $p59^{fyn}$ (Quill et al., 1992; Cho et al., 1993). Recently, Fields et al. have shown that in T cell clonal anergy the state of unresponsiveness is maintained at least in part by a block in the activation of $p21^{ras}$ (Fields et al., 1996). The current model (Schwartz, 1997) propose that this block in Ras activation leads to a decrease in signaling through the ERK and JNK pathways (Li et al., 1996), resulting in a decrease in c-Fos and JunB induction (Mondino et al., 1996). As a consequence, there is a failure to phosphorylate the activator protein (AP)-1 heterodimers required for IL-2 gene transcriptional activation.

As most of the cells in the peripheral tissues of the body are poor antigen-presenting cells and therefore are unlikely to provide signal two, the "two signal model of lymphocyte activation", provided one significant explanation for the induction of peripheral tolerance. For example, T cells with specificity for a peptide derived from myoglobin would be rendered anergic after encountering self MHC plus myoglobin peptide on a muscle cell that cannot provide signal two. Furthermore, it has been proposed that the failure to generate antitumor response against antigens expressed by tumors of nonhematopoietic origin is related to the inability of these cells to provide adequate costimulation because ***most of these tumors lack the critical signal 2*** necessary to induce an immune response (Ostrand-Rosenberg, 1994).

Needless to say, there has been considerable interest in the identification of the receptor-ligand pairs that are present on APCs and T cells that might mediate this signal 2. While there have been reports of several cell surface molecules (particularly adhesion molecules) that might deliver a second signal, there has been a large body of evidence implicating a particular family of cell surface molecules known as **B7** present on APCs as important in delivering costimulatory signals to T cells (Gimmi et al., 1991; Reiser et al., 1992: Freeman et al., 1993a). These molecules bind to a receptor on T cells called CD28. When anti-CD28 antibodies are added to the *in vitro* T cell anergy system described by Jenkins and Schwartz (T cells mixed with fixed APCs plus antigen), CD28 molecules on the T cell clone become crosslinked at the time of T cell receptor engagement. This result is *activation* rather than anergy, implying that CD28 crosslinking generated the missing signal 2 that the fixed APCs could not provide (Harding et al., 1992). Also, if monovalent anti-CD28 Fab′ fragments (which cannot crosslink CD28 but rather will block interactions between B7 on the APC and CD28 on the T cell) are added to an *in vitro* system that contains fresh APCs plus antigen, T cell clones become anergized rather than activated (reviewed in Allison, 1994). The B7-CD28 interaction has also been shown to be necessary and sufficient costimulation for $CD8^+$ CTL generation in the absence of exogenous help (Harding and Allison, 1993; Azuma et al., 1993). Therefore, these studies *strongly supported the B7-CD28 interactions as a likely candidate for signal 2*.

The relative importance of CD28 and B7 in mediating costimulation was clarified in gene knockout experiments in mice. CD28-deficient mouse lines demonstrated pronounced and specific immune defects. Although T cell development was normal in the CD28 knockout mice, the peripheral T cells had impaired lymphokine secretion after stimulation with mitogens. In addition, there were defects in the humoral immune response of these mice likely secondary to diminished T cell help to B cells (Shahinian et al., 1993). In contrast to the clear phenotype of the CD28 knockout mice, B7 knockout mice were found to have virtually no immune defects (Freeman et al., 1993b). These animals led to the discovery that there was a second ligand for CD28 found on APCs (Freeman et al., 1993a). The first B7 family member was therefore termed B7-1 (CD80) and the second one was denominated B7-

2 (CD86). Although several hypotheses have been put forward to ascribe differential functions for B7-1 vs. B7-2, this issue remains unresolved (Schweitzer et al., 1997).

The expression of B7-1 and B7-2 has been shown to be relatively restricted to antigen-presenting cells. Among these cells a hierarchy with regard to the expression of B7 has been found. Moreover, this expression of B7 molecules correlates with their potency to stimulate T cells. Some cells, such as dendritic cells, are highly potent at inducing T cell responses *in vitro* and they have been shown to express the highest levels of B7 molecules. This potency is less dramatic in activated B cells and macrophages and falls off further in resting B cells (Fuchs and Matzinger, 1994). Finally, cells of nonhematopoietic lineage are very poor at inducing primary T cell responses and in fact they failed to express B7 molecules. B cells are particularly of interest with regard to the mechanisms involved in the regulation of B7 expression. It has been demonstrated that resting B cells express low levels of B7-1 and B7-2, but that these are upregulated after B cell activation with lipopolysaccaride, crosslinking of surface immunoglobulin (Lenschow et al., 1994), and CD40-CD40-ligand interactions (Grewal et al., 1996; Yang and Wilson, 1996). The regulated expression of costimulatory molecules by APCs appears to be one component of the "cross-talk" that occurs during T cell–APC interactions that result in activation of an ensuing immune response.

A second ligand for B7-1 and B7-2 is a molecule expressed on activated T cells known as *cytotoxic T lymphocyte antigen-4* or CTLA-4 (Brunet et al., 1987; Linsley et al., 1991b). In contrast to CD28, which is expressed on most resting T cells, *CTLA-4 expression occurs only after T cell activation*. Interestingly, the binding affinity of CTLA-4 for B7-1 and B7-2 is roughly twenty-fold higher than that seen for CD28-B7 interactions (June et al., 1994). The high affinity of the interaction of CTLA-4 with the B7 family members was taken advantage of by Linsley and co-workers, who engineered a soluble version of CTLA-4 that was fused to the constant region of the human immunoglobulin molecule (CTLA-4-Ig). This fusion protein when expressed in a secreted form has been a very useful tool in demonstrating the *in vivo* effects of blocking B7-CD28 interactions. These studies demonstrated that treatment of mice *in vivo* with CTLA-4-Ig strongly inhibits T-cell-dependent antibody responses (Linsley et al., 1992) and furthermore permits the long-term acceptance of xenografted pancreatic islets (Lenschow et al., 1992). Although it was initially thought that CTLA-4 may represent an additional costimulatory molecule, subsequent studies involving CTLA-4 knockout mice (Tivol et al., 1995; Waterhouse, 1995), and specific *in vivo* blockade with antibodies against CTLA-4 has strongly implicated this molecule as a downregulator of T cell activation (Walunas et al., 1994; Krummel and Allison, 1995; Karandikar et al., 1996; Perrin et al., 1996; Chambers and Allison, 1997). Furthermore, blockade of the inhibitory effects of CTLA-4 with anti-CTLA-4 antibodies *in vivo* has been shown to result in the rejection of B7-1 positive and B7-1 negative colon adenocarcinoma, even when they were preestablished in experimental models (Leach et al., 1996). Recently, Perez et al., using T cells expressing a transgenic TCR specific for an OVA peptide, have shown that the induction of peripheral T cell tolerance *in vivo* requires CTLA-4 engagement (Perez et al., 1997). Therefore, in addition to its inhibitory role during T cell activation, CTLA-4 may play a key role in the regulation of peripheral tolerance (reviewed in Bluestone, 1997).

2. In Vivo *Models of Peripheral Tolerance*

Although the *in vitro* models of anergy described above provided a conceptual framework for how tolerance might be maintained to antigens expressed by peripheral tissues, one of the criticism of these early studies was that they were performed with T cell clones that may have been selected for their ability to survive prolonged passage *in vitro*. Ultimately, *in vivo* experiments were required to further understand and expand the knowledge in this field. A large number of these experiments have taken advantage of the ability to selectively express a defined protein antigen exclusively in the islets of Langerhans in the pancreas. This is accomplished by expressing the gene of interest under the regulation of an insulin promoter ("RIP"–rat insulin promoter).

Transgenic mice made in this fashion express the transgene on the islet cells of the pancreas but no other tissues. In these experiments, the readout is typically a histologic examination of the pancreatic islets looking for inflammation as well as monitoring the animal's blood sugar levels for evidence of the development of diabetes that would occur when the islet cells are destroyed by inflammatory cells (Lo et al., 1988).

The use of transgenic mouse models, has therefore allowed the description of different mechanisms for generation of tolerance to peripheral self-antigens *in vivo*. Several potential outcomes can result from the interaction of T cells with antigen expressed peripherally:

a. Anergy
b. Peripheral clonal deletion or "exhaustion"
c. Ignorance
d. Clonal diversion
e. Suppression and/or suppressor cells

a. Anergy

The first of these *in vivo* studies expressed the MHC class II molecules I-E under control of the insulin promoter in a mouse strain that was otherwise negative for I-E expression (Lo et al., 1988). These studies took advantage of the knowledge that T cells expressing the Vβ17 family of T cell receptors have reactivity to a murine retroviral superantigen that binds to I-E. Because monoclonal antibodies were available that could stain Vβ17-bearing cells, it was possible to examine the fate of these cells in the transgenic mice expressing I-E exclusively on the pancreatic islets. What was found was that these animals did not develop infiltrates in the islet cells or diabetes. Instead, the T cells of these mice were tolerant to I-E, and the islet cells were not rejected. *However, Vβ17-bearing T cells were not deleted.* Instead they circulated in an anergic state, that is, they were not able to be activated. These results suggested that T cells with specificity for I-E plus the murine retroviral superantigen received signal 1 after encountering the pancreatic islets but failed to receive signal two from these "non-professional" APCs, the result being tolerance. Subsequent experiments expressed the allogeneic MHC class I gene H-2K^b under the insulin promoter. These animals failed to develop immune-mediated rejection of the islets cells, and spleen cells taken from these mice could not generate H-2K^b-specific allogeneic CTL *in vitro*. In contrast, thymocytes taken from these mice were able to develop K^b-specific CTL, suggesting that the tolerance generated was indeed *peripheral tolerance* and required newly generated thymic emigrants to encounter the antigen on the pancreatic islets for the induction of unresponsiveness (Morahan et al., 1989).

b. Peripheral Clonal Deletion or "Exhaustion"

Superantigens are proteins that stimulate T cells by bridging the nonpolymorphic regions of MHC class II molecules on an APC with the β chain of the T cell receptor. Individual superantigens, which are the products of bacteria (e.g., staphilococcus enterotoxin B) or viruses (e.g., MMTV), bind to T cell receptors utilizing particular TCR Vβ families, resulting in the delivery of a strong signal to significant number of T cells bearing the Vβ product. If the superantigen is present in the thymus (such as Mls antigen, which was found to be a product of MMTV), this results in deletion of all T cells having a TCR with that particular Vβ chain. *In vitro* studies, mixing lymphocytes from a MMTV negative strain (Mls^b) with lymphocytes from a strain expressing the superantigen (Mls^a) results in intense proliferation of the Vβ6$^+$ T cells of the Mls^b lymphocytes. This system was subsequently used to examine whether the mechanisms of clonal deletion were also operative in the induction of peripheral tolerance. MMTV negative (Mls^b) strain mice were thymectomized, so that any effects measured would reflect events that were independent of thymic or central tolerance. These mice were then injected with MHC-matched lymphocytes that were from a strain that expressed the superantigen (Mls^a). What was found was that early after injection (4 days), there was a sharp *increase* in the number of T cells expressing Vβ6, whereas at later time points there was a dramatic *elimination* of Vβ6$^+$ T cells (Rammensee et al., 1989). When examined for functional responses to Mls^a lymphocytes *in vitro*, at day 4 Vβ6$^+$ T cells were

hyperproliferative, whereas at later time points the responses were blunted. Similar responses (initial expansion followed by deletion) were seen in a model of Vβ8+ T cells that respond to staphylococcal enterorotoxin B *in vivo* (McDonald et al., 1991). More recently, Zinkernagel's group, using TCR-transgenic mice specific for lymphocytic choriomeningitis virus (LCMV), have shown that virus persistence in immunocompetent mice was related to the *exhaustion* of the specific antiviral CD8+ cytotoxic T cells. Once again, the pattern of response was an initial vigorous expansion of these cells followed by a rapid decline to undetectable levels by day 15 after viral challenge (Moskophidis et al., 1993). Taken together, these results essentially described a form of *activation-induced peripheral clonal deletion* to a strong antigenic stimulation. When confronted with this challenge, the immune system initially reacts vigorously, but then somehow limits the extent of the response and eliminates T cells that had become activated (Webb et al., 1990). These results were reminiscent of older studies that described tolerance induction by injection of large amounts of antigen that would otherwise result in priming at lower doses (Weigle, 1973).

The molecular basis for this mechanism has been identified recently. Two mutant mouse strains known as *MRL/lpr* and *gld* have long been studied as models for autoimmune diseases such as lupus erythematosus. As they age these animals develop antibodies to several self-antigens, leading to immune complex deposition in the kidneys as well as other manifestations of autoimmunity. They have also been shown to accumulate autoreactive T cells. In addition, starting at about 6 to 8 weeks of age they develop a lympho-proliferative syndrome characterized by progressive enlargement of the lymph nodes and other secondary lymphoid tissues (Cohen and Eisenberg, 1991). Recently, it has been found that MRL/lpr mice have a mutation in a gene known as *Fas* (Watanabe-Fukunaga et al., 1992), while gld mice are mutant for a gene encoding *Fas ligand* (Takashi et al., 1994). Fas encodes a transmembrane protein that is a member of the tumor necrosis factor (TNF) and nerve growth factor (NGF) receptor families (Yonehara et al., 1989) and is expressed on a variety of cell types, including activated T cells. When crosslinked, either experimentally with an antibody or physiologically by its ligand, Fas delivers a signal to the cell triggering *programmed cell death or apoptosis* (Trauth et al., 1989, Rouvier et al., 1993). In contrast to Fas, Fas ligand is almost exclusively expressed by activated T cells and cells of the so-called immune privileged sites (Streilein, 1995). Crosslinking of Fas on target cells by activated cytolytic T cells expressing Fas ligand is one of the two known pathways by which cytolytic T cells kill their targets (Kagi et al., 1994; Lowin et al., 1994). A model has been proposed that T cells upregulate both Fas and Fas-ligand during activation such that when they reach a certain density they actually kill each other ("fratricide"), thus limiting the magnitude of a given immune response (Russell et al., 1993; Vignaux and Golstein, 1994; reviewed in Russell, 1995). The mutations found in MRL/lpr or gld mice disarm this counterregulatory mechanism and result in excessive lymphoproliferation and accumulation of autoreactive T cells that would have otherwise been eliminated after initial activation. Proof that Fas-Fas ligand interactions account for the clonal exhaustion described above came by the crossing of a TCR transgenic mouse with an MRL/lpr mouse (Singer and Abbas, 1994). Whereas injection of large quantities of the antigen recognized by the transgenic T cells resulted in deletion of these cells in the single TCR transgenics, TCR transgenic mice crossed to MRL/lpr mice, which lack the counterregulatory Fas molecule, developed progressively increasing numbers of transgenic T cells when injected with antigen due to the lack of the expression of the counter-regulatory molecule Fas.

Recently, Fas-Fas ligand interactions have been shown to play a role in one other, somewhat specialized form of peripheral tolerance—so-called *immune privileged sites*. It has long been known that allogeneic tissue grafts implanted in the testis or in the anterior chamber of the eye fail to be rejected. It has now been found that these sites constitutively express Fas ligand (Griffith et al., 1995; Belgran, 1995). Accordingly, T cells that traffic to these tissues and become activated are presumably quickly killed off, a mechanism that may have evolved to limit the damage that these key organs would encounter if otherwise inflammation occur.

c. "Ignorance"

The models of tolerance presented above require the T cells to encounter self MHC plus sufficient quantities of peptide derived from self-antigen to deliver signal one in the absence of signal two. An alternate explanation for how the immune system may fail to respond to self-antigens is that *signal one is never adequately delivered.* The situation could arise if the density of MHC plus antigenic peptide derived from self proteins is below a threshold necessary to deliver the first signal to potentially autoreactive T cells. Alternatively, this could occur if T cells with specificity for tissue-specific antigens simply never trafficked to sites where they would encounter the antigen in the first place. The outcome of this mechanism of tolerance would be neither activation nor anergy, but rather what has been dubbed "ignorance" (the immune system's version of "don't ask, don't tell"). Rammensee and co-workers reported the ability to isolate CTL from mice that were specific for a variety of self proteins such as beta-2 microglobulin, hemoglobin, and a pool of normal total liver proteins. These CTL could recognize syngeneic targets pulsed with the appropriate peptide antigens derived from these normal self proteins, but interestingly failed to recognize targets that endogenously expressed these proteins, suggesting that the endogenous levels of the appropriate peptides on MHC was too low for recognition (Schild et al., 1990). This analysis suggested that autoreactive CTL are not deleted or anergic *in vivo* but rather fail to become activated when they encounter their peptide MHC complex on peripheral tissues expressed at physiologic concentrations. A second set of studies that lends support to the ignorance model placed the gene for the lymphocytic choriomeningitis virus nucleoprotein (LCMV-NP) gene under the regulation of the rat insulin promoter. These animals expressed NP in the islets of Langerhans but failed to express the gene in any other tissue. These animals fail to develop infiltrates in the islet cells and did not develop diabetes. However, when LCMV-specific CTL were adoptively transferred, these cells migrated to the islets of Langerhans and destroyed the tissue. More importantly, when these transgenic mice were given a systemic challenge with live LCMV virus, the vast majority developed diabetes and infiltrating mononuclear cells in the islets. When these infiltrating cells were characterized *in vitro* they were found to largely be $CD8^+$ T cells that had specificity for LCMV-infected targets. Consequently, this model indicates that expression of viral nucleoprotein as a tissue-specific self-antigen failed to induce an irreversible tolerant state, and infection later in life with a pathogen expressing this antigen results in priming of antigen-specific CTL and destruction of the peripheral tissue (Oldstone et al., 1991). A related study (Ohashi et al., 1991) crossed transgenic mice expressing LCMV glycoprotein (GP) in the pancreatic islets with T cell receptor transgenic mice where the TCR was specific for LCMV-GP. Remarkably, while over 50% of the T cells in the double transgenic mice were $CD8^+$ CTL specific for the LCMV glycoprotein, diabetes failed to develop unless these mice were challenged with the live LCMV virus. There was no evidence of elimination of the LCMV-specific transgenic T cells in animals expressing LCMV-GP on the islets of Langerhans. These results suggested that neither deletion or anergy was responsible for tolerance in this model.

The importance of costimulatory signals such as B7 in differentiating between tolerance and reactivity was formally demonstrated by an extension of the above transgenic experiments. Transgenic mice expressing B7 under control of the rat insulin promoter were crossed with the RIP-LCMV glycoprotein transgenic. In these animals, antiviral CTL were activated without viral infection and spontaneous diabetes occurred, in contrast to the single transgenic expressing LCMV glycoprotein alone. Although this study does confirm that B7-1 expression can enhance T cell activation to a defined antigen, it is interesting to note that single transgenic mice expressing B7-1 under the rat insulin promoter alone (without LCMV antigens) failed to develop diabetes, and that reasonably high levels of the model antigen (LCMV glycoprotein) were required to see induction of autoimmunity in the double transgenic mice. Presumably, the level of expression of naturally occurring islet cells specific proteins that might serve as targets for autoimmunity in the single B7-1 transgenics was too low to result in the induction of autoimmunity, even though costimulation was provided (Guerder et al., 1994).

d. Clonal Diversion

Recently, there has been increased appreciation for the fact that T cell activation may result in markedly different outcomes depending on the patterns of cytokines that are produced. In particular, cytolytic T cell responses are thought to be favored by the production of so-called "T helper 1" (Th1) cytokines such as gamma-interferon and IL-2, while antibody production and diminished cell-mediated responses are associated with "T helper 2" (Th2) cytokine responses such as IL-4, IL-5, and IL-10 (Mosmann and Coffman,1989; reviewed in Seder and Paul, 1994). The potential importance of the pattern of cytokine response to the development of tolerance vs. autoimmunity was underscored in a study by Lo and colleagues (Scott et al., 1994). These investigators created transgenic mice expressing influenza hemagglutinin under the rat insulin promoter. In addition, they generated T cell receptor transgenic mice where the α and β chain of the TCR were derived from a CD4 clone specific for influenza hemagglutinin presented by MHC class II molecules. The initial cross of these mice generated double trangenics that, like the models described above, failed to demonstrate evidence of diabetes or inflammatory islet cells infiltrates. No evidence of clonal deletion or anergy was seen in the hemagglutinin-specific T cells examined from the double transgenic mice. However, when these animals were backcrossed to a B10.D2 background (having the same haplotype, H-2^d, as the original mice but different background genes) these animals *did* develop spontaneous diabetes. Previous studies had demonstrated that B10.D2 mice have a predisposition to respond to certain pathogens with a Th1 pattern of cytokines, and BALB/c mice tend to respond in a Th2 pattern (Sadick et al., 1987; Heinzel et al., 1989). In very elegant experiments T cells were taken from the double transgenic mice and crossed to each of these two genetic backgrounds and examined for cytokine release. It was found that the diabetes prone B10.D2 animals were indeed making Th1 cytokines in response to hemagglutinin, whereas the diabetes-resistant BALB/c double transgenics were producing Th2 cytokines. Analysis of activation markers from T cells obtained from both the resistant (BALB/c) and diabetic animals (B10.D2) demonstrated that these T cells had in fact been activated, *but that the Th-2 response resulted in tolerance while the Th-1 response resulted in diabetes*. These observations have suggested that one mechanism of tolerance may be the "**diversion**" of the cytokine responsive T cells away from that which supports cell-mediated tissue destruction toward a response that favors tolerance. Currently, there is intense interest in identifying the genetic factors that mediate the tendency toward responding one way or another.

e. Suppressor Cells and Immunosuppression

In the early 1970s, one of the emerging theories to explain self-tolerance was through the existence of cells termed "suppressor cells" capable of exerting immunosuppressive effect on other cells of the immune system rendered them unresponsive to self-antigens or exogenous antigens in an antigen-specific manner. This concept was based on the classic experiments of Gershon and Kondo using a high-dose-tolerance experimental model (Gershon and Kondo, 1971). These investigators mixed cells obtained from tolerant animals with cells from naive animals *in vitro* and these mixtures were then adoptively transferred into T-cell-depleted animals. What was found was that the recolonized animals were still unresponsive to the exogenous antigen when this was introduced in a fashion that would otherwise result in an active immune response in the naive animal. As half of the cells from the transferred mixture were from a nontolerant donor, it was postulated that the cells from the tolerant donor were "*actively suppressing*" the cells from the naive donor, leading to their unresponsiveness. The phenotype of these suppressor cells was initially thought to be related to $CD8^+$ T cells, but this concept was abandoned after many attempts to clone suppressor cells *in vitro* or to dissect mechanisms for suppression failed to provide any experimental support to this hypothesis.

In recent years, however, a renewed interest in the phenomenon of active immunosuppression has occurred, as interesting data have been provided by several groups. It has been found that $CD8^+$ T cells are able to recognize MHC class I plus peptides derived from specific T cell receptors on autoreactive effector T cells, leading to

their elimination and the suppression of autoimmunity (Wraith et al., 1989). Also the finding of two subsets of CD4+ T cells with different pattern of cytokine production has provided support that under certain experimental conditions CD4+ T cells can function as suppressor cells through the production of inhibitory cyto-kines. In fact, IL-4 and IL-10 produced mainly by Th-2 cells have been shown to inhibit the activation of macrophages to kill intracellular leishmania parasites (Sadick et al., 1990). Moreover, these cytokines inhibit the *de novo* generation of Th1 cells, leading to a polarized phenotype (Fiorentino et al., 1991). Conversely, IFN-γ produced by Th-1 cells inhibits the development of the Th-2 phenotype (Gajewsky and Fitch, 1988). Interestingly, when T cells already committed to the Th2 pathway are transferred into an animal that was infected with leishmania, they would suppress the generation of an effective immune response against leishmania, rendering these animals susceptible to be killed by this infection.

IV. TOLERANCE AND CANCER

A. Central Tolerance and Hematologic Malignancies

A unique aspect of the immune response to hematopoietic cancers when compared with solid tumors is the potential role of central tolerance. As mentioned previously, potentially autoreactive thymocytes are deleted with high efficiency when they express T cell receptors having a high affinity for antigens presented by bone marrow-derived elements of the thymus (Marrack et al., 1988). For diseases such as chronic myelogenous leukemia (CML) involving multiple hematopoietic lineages, including bone marrow derived thymic interdigitating cells (IDCs), it is likely that T cells with specificity for tumor-specific antigens expressed by these cells will be deleted (negative selection). Carrying the example of CML one step further, a specific prediction that follows from this argument is that patients who have been in the chronic phase of this disease for some time will be incapable of generating T cells specific for neo-antigens, such as epitopes derived from the fusion protein p210 encoded by the bcr-abl rearrangement, which constitutes the molecular hallmark of this disease. However, patients who have only recently acquired the disease may have p210 reactive T cells in the periphery that exited the thymus before the organ became "colonized" with bone marrow-derived cells expressing the neo-antigen. In addition, patients in the chronic phase of CML are often "chimeras", that is, their hematopoietic elements are a mixture of normal and bcr-abl rearranged cells. Therefore, it is possible that such T cells could escape negative selection if the ratio of normal to bcr-abl rearranged APCs in the thymus were favorable (Levitsky, 1996a).

An aspect of central tolerance with far greater implications for the nature of immune responses to hematopoietic cancers is the thymic deletion of T cells specific for hematopoietic differentiation antigens. Recent evidence has revealed that tissue-specific differentiation antigens are frequently recognized by T cells from patients with melanoma. These are not unique "tumor-specific" neo-antigens such as might arise from a mutation, but rather are antigens derived from normal proteins involved in the production of pigment by melanocytes (Houghton, 1994). Such proteins are in essence *lineage-specific tissue differentiation antigens* expressed by tumors arising from these tissues. Under normal circumstances, T cells would have little occasion to encounter these antigens. However, once inflammation is present, such as might occur with necrosis of the tumor or vaccination, T cell priming can occur against these tissue-specific antigens that can now serve as perfectly good tumor-rejection antigens. In sharp contrast, many of the proteins that are common to hematopoietic cells and their transformed counterparts, which distinguishes them from other tissues, are likely to be expressed by the cells in the thymus that mediate negative selection. A prediction based on this argument is that hematopoietic cancers will have a more limited spectrum of potential tumor-rejection antigens, as the host is likely to be tolerant to tissue differentiation antigens expressed by these tumors. However, central tolerance seems not to be an "all or none" phenomenon, as ascertained by recent studies that have challenged the initial concept of central tolerance as the predominant mechanism of establishing self-tolerance (Amakawa et al., 1996; Rocha et al., 1993, Bluestone, 1997). Thus, the

possibility exists that T cells specific for tissue differentiation antigens expressed in hematologic cancers may escape the normal mechanisms of central tolerance and therefore might be capable of responding to immune-enhancing therapeutic approaches (i.e., vaccination). Moreover, tumors derived from other hematopoietic elements (such as B cell lymphomas) still express more highly restricted, lineage-specific antigens that are unlikely to be presented in the thymus, suggesting that at least in theory an effective generation of T-cell-mediated immunity against these antigens might be a feasible approach to undertake.

B. Peripheral Tolerance and Cancer

1. Anergy

As previously described, full activation of resting T cells requires not only an antigen-specific signal provided by engagement of the T cell receptor with the appropriate peptide/MHC complexes, but also requires a second signal delivered by specialized APCs that upregulate the expression of "co-stimulatory" molecules such as B7-1 and B7-2 that engage CD28 on the T cell (June et al., 1994; Allison et al., 1995). Engagement of the T cell receptor in the absence of a co-stimulatory second signal result in T cell anergy; this state of unresponsiveness being maintained even if both signals are provided in a subsequent encounter with the antigen (Jenkins et al., 1987).

As most tumor cells are poor antigen-presenting cells that are incapable of expressing co-stimulatory molecules (but usually express MHC class I molecules and can be induced to express MHC class II), the above paradigm predicts that tumor-specific T cells would be rendered anergic after encountering tumor antigen on the MHC molecules of the cancer cell. In support of this hypothesis, several groups have demonstrated that tumor immunity can be enhanced by the provision of costimulatory signals. It has been shown that strong antitumor T cell responses can be generated with vaccination with tumor cells transfected to express B7-1. This strategy resulted in rejection of the B7-1-transfected tumor cells and the generation of immunity to rechallenge with the parental B-7 negative tumor cells (Chen et al., 1992; Towsend and Allison, 1993; Baskar et al., 1993). In other words, this strategy attempts to turn "non-professional" APCs into somewhat better ones, so that now tumor cells can provide the T cell with both "signal one" through TCR crosslinking by tumor MHC/peptide complexes and "signal two" through CD28 crosslinking by the transfected B7-1 molecule.

The mechanisms involved in the generation of effective antitumor responses against B7-1$^+$ tumor cells appears to be related to the predominant activation of CD8$^+$ T cells (Harding and Allison, 1993; Towsend et al., 1994). However, the role of CD4$^+$ T cells has been highlighted in the SA1 murine sarcoma model, as well as in a subline of K1735 melanoma cells. In these models, B7-1-transfected tumor cells induced CD4$^+$ and CD8$^+$ T cell responses and both cell subsets were shown to play a significant role in the induction of tumor regression (Baskar et al., 1995; Li et al., 1994). As postulated by Allison, it would be beneficial in having CD4$^+$ T cells involved in the antitumor response generated by B7-1-transfected tumor cells. The presence of tumor-specific CD4$^+$ T cells, elicited by antigen-MHC class II complexes either on the transfected tumor cell or host APCs, may result in better activation of cytotoxic T cells and therefore a stronger and sustained antitumor response (Allison et al., 1995). Other mechanisms that have been postulated to account for the immunity to B7-1$^+$ tumor cells are the capacity of cytotoxic T cells to respond to an increased number of antigenic peptides, a phenomenon known as *epitope spreading* (Johnston et al., 1996). Furthermore, vaccination with B7-1-transfected tumor cells has been postulated to result in the liberation of tumor antigens that then can be taken up, processed, and presented by host APCs, resulting in T cell priming (Huang et al., 1994; Huang et al., 1996).

The discovery of a second member of the B7 family (B7-2) prompted several investigators to asses the effectiveness of B7-2 transfected tumor cells in eliciting antitumor immunity. Yang et al., using B7-2 transfected P-815 tumor cells, have shown that these cells elicit tumor regression and that CD8+ T cells were responsible for the antitumor effect observed. Furthermore, when these animals were challenged with wild-type P-815 tumor, a systemic immunity was also observed

(Yang et al., 1995). Similarly, in a murine MC38 carcinoma model, it was found that cells transduced with recombinant vaccinia virus vectors containing either B7-1 or B7-2 genes generate a strong antitumor immunity leading to their rejection when these transfected cells were injected into naive animals (Hodge et al., 1994).

However, despite these encouraging results it was rapidly realized that the expression of either B7-1 or B7-2 was not sufficient to induce regression in a variety of tumor models. In fact, immunization with A20 lymphoma cells engineered to express elevated levels of B7-1 failed to generate systemic immunity (Levitsky et al., 1996b). Given that B-cell lymphomas represent MHC class II-positive tumors derived from cells capable of providing the necessary signals for T cell activation, it is perhaps surprising that strategies aimed at enhancing direct antigen presentation by the tumor, such as the transfection with B7-1 gene, were ineffective in this tumor model. Moreover, these results demonstrated that despite achieving a level of B7-1 expression that dramatically enhances APC function *in vitro*, this was not enough to prime a sufficient anti-tumor response *in vivo*. One explanation for these findings may relate to the relative lack of immunogenicity of A20 tumor cells, as it has been shown that the systemic immunity induced by B7-1-transfected tumor cells appears to correlate with the inherent immunogenicity of the tumor (Chen et al., 1994). In fact, immunization with the relatively nonimmunogenic B16F10 melanoma transfected with B7-1 results in the rejection of the live transfectant but fails to prime systemic immunity to the parental tumor (Wu et al., 1995). Although MHC class-I-restricted CTL can be directly primed by the B7-1-transfected tumor, this mechanism appears to be far less efficient than cross-priming of CTL by host APCs that have taken exogenous tumor antigens (Huang et al., 1996). Another factor that may account for the limited efficacy of immunizing with B7-transfected tumor cells is that other as yet undefined signals may be required for efficient T cell activation *in vivo*.

Although several vaccines strategies have been shown to be capable of priming potent antitumor immune responses when delivered to tumor-free recipients, the impact of vaccination is limited in the tumor-bearing host. One explanation for this observation is that antigens presented by a progressively expanding tumor cell population may result in the *induction of antigen-specific T cell tolerance*. Using T cell receptor (TCR) transgenic mice specific for a model tumor antigen expressed on A20 B cell lymphoma, recently we have obtained direct evidence supporting the concept of *tumor-induced antigen-specific tolerance* (manuscript submitted). Based on the adoptive transfer system reported by Kearny and Jenkins (Kearney et al., 1994), we have been able to monitor *in vivo* the fate of an identifiable population of transgenic TCR $CD4^+$ T cells specific for hemagglutinin (HA) amino acids 110 to 120 restricted by $I\text{-}E^d$. The A20 B cell lymphoma has been transfected to express influenza hemagglutinin (A20HA), and we have selected clones expressing low levels that are nonetheless recognized by the anti-HA/$I\text{-}E^d$ transgenic $CD4^+$ T cells *in vitro*. Therefore, the availability of a identifiable population of T cells with a defined specificity and a tumor expressing the antigen recognized by these cells has enabled us to examine the frequency and function of tumor-specific T cells during tumor progression.

It should be point out that the expression of HA did not alter the immunogenicity of A20 lymphoma cells, as intravenous injection of BALB/ c mice with either A20 wild-type or A20HA resulted in tumor growth with similar kinetics. Interestingly, the kinetics of tumor growth was not affected by the adoptive transfer of anti-HA/$I\text{-}E^d$ transgenic T cells given 9 days after tumor challenge. Studies of explanted tumors have shown that in no case was loss of antigen by A20 cells responsible for the outgrowth of A20HA tumor. Mice bearing A20HA tumors showed an initial expansion of clonotype positive T cells, which was followed by a decline in this cell population as the lymphoma progressed. Furthermore, an activation phenotype ($CD44^{high}$, $CD45^{low}$, $CD62^{low}$) was displayed by these cells during their initial expansion. Despite these changes, antigen-specific $CD4^+$ T cells from A20HA-bearing mice were found to be functionally impaired. In fact, in A20HA-bearing mice, this cell population failed to be primed by immunization with a recombinant vaccinia virus encoding the model antigen. Evidence for this included (1) lack of clonotypic expansion of these $CD4^+$ T cells when they reencounter antigen *in vivo*, (2) failure to produce

IL-2, IFN-γ, and to proliferate in response to stimulation with HA-peptide *in vitro*. These findings pointed out that antigen-specific CD4+ T cells are rendered tolerant, and more importantly these alterations in T cell function occur early in the course of tumor-T cell interaction and significantly preceed the development of a more generalized state of immunosuppression. Interestingly, the proliferative response of transgenic T cells from A20HA-bearing mice to HA peptide was partially restored in the presence of exogenous IL-2, reminiscent of the findings observed in *in vitro* models of T cell anergy (Jenkins et al., 1987). Similar restoration of the proliferative response of tolerized transgenic T cells was observed when IFN-γ was used in combination with HA-peptide to stimulate these T cells *in vitro* (Sotomayor et al., manuscript in preparation).

2. Clonal Deletion

Peripheral clonal deletion of T cells was one of the earliest counterregulatory mechanisms to be identified as playing an important role in the maintenance of tolerance and homeostasis of the immune system. After challenge with a strong antigenic stimulation, the immune system mounts an initial vigorous response that is followed by a rapid elimination of activated T cells, a process that is also known as *activation-induced cell death* (AICD). As noted above, recently it has been shown that *Fas-Fas ligand* interactions mediate this downregulation of T cell responses by induction of apoptosis of T cells in the periphery (Russell et al., 1995). Interestingly, some types of tumor cells may have evolved to exploit this mechanism as a means to evade immunologic rejection. Tschopp's group has recently found that human melanoma cells express Fas-ligand, which may contribute to the immune privilege of these tumors (Hahne et al., 1996). In a series of elegant experiments, these investigators found that these melanoma cells were cytolytic and killed Fas-positive A20 lymphoma cells *in vitro* via a Fas-Fas ligand-dependent pathway. Moreover, *in vivo* injection of Fas-ligand-positive mouse melanoma cells into fully allogeneic recipients results in a rapid tumor growth. In sharp contrast, allogeneic Fas-deficient *lpr* mice, which lack Fas-positive immune effector cells, showed a slower growth of the Fas-ligand expressing tumor when compared with wild-type animals. These results were explained on the basis that the immune effector cells in the *lpr* mice are less sensitive to FasL-dependent apoptosis and therefore are capable of generating an efficient allogeneic response leading to the observed delay in tumor growth. This initial observation in melanoma cells was soon extended to other tumor types. In fact, the analysis of hepatocellular carcinoma cells obtained from 22 patients showed an increased expression of Fas-ligand in these tumor cells that was associated with a decreased or no expression of Fas receptor (Strand et al., 1996). Taken together, the expression of Fas-ligand by tumor cells may confer an advantage to the tumor, which is now capable of inducing immune tolerance through clonal deletion of those Fas-positive reactive immune cells that otherwise are capable of mediating antitumor responses.

In a plasmacytoma model, Bogen has also found that peripheral T cell tolerance is associated with clonal deletion of CD4+ T cells specific for a monoclonal immunoglobulin idiotype secreted by these B cell tumors. This clonal deletion was induced in a dose-dependent fashion by the idiotype protein being secreted in the serum and become more significant as the tumor burden increases (Bogen, 1996). These results are in agreement with studies that have shown tolerance induction by injection of large amounts of soluble antigens that would otherwise result in priming at lower doses (Weigle, 1973; Liblau et al., 1996). However, the mechanism(s) involved in the clonal deletion observed in this model remains to be elucidated.

3. Ignorance

Different studies have provided support for the ignorance model as a mechanism of tolerance to self proteins (Ohashi et al., 1991; Oldstone et al., 1991). It has been proposed that "ignorance" develops because the density of MHC plus antigenic peptide derived from self proteins is below a threshold necessary to trigger an adequate "signal one" to potentially autoreactive T cells. An alternate explanation is that these autoreactive T

cells never traffic to sites where they would encounter the antigen in the first place. As far as we know, no model of ignorance has been shown to occur in the tumor setting. However, it is not hard to speculate that for certain solid tumors, T cells with specificity for tumor antigens simply fail to encounter the antigen, resulting in neither priming nor anergy. In contrast, failure of T cell-tumor interaction seems far less plausible for hematologic cancers. After all, these malignant cells reside in the very lymphoid organs in which T cell immunity is generated. In fact, using the previously described *in vivo* model that allowed us to follow the fate of transgenic T cells specific for a model tumor antigen expressed by A20 lymphoma cells, we have found that "ignorance" seems not to be responsible for the observed immune tolerance. This was ascertained by (1) presence of both A20 tumor cells and transgenic T cells in the spleen of the analyzed animals, (2) phenotypic changes associated with antigen recognition were found in HA-specific transgenic T cells obtained from A20HA-bearing mice ($CD44^{high}$, $CD45rb^{low}$, and $CD62^{low}$) when compared with non-tumor-bearing mice. Whether this recognition and activation of T cells occur in other *in vivo* models of hematologic malignancies will need to be studied further before the mechanism of ignorance can be ruled out as operative in the induction of tolerance in cancers of hematologic origin.

4. Clonal Diversion

Differentiation of $CD4^+$ T cell into Th1 or Th2 cells is a crucial event in determining whether a cellular or humoral response is generated by the immune system. The factor(s) that determine this differentiation are not fully understood; however, it has become clear that the pattern of cytokine production that characterizes a Th1 vs. a Th2 response has a significant impact on the overall character of these responses (Mosmann and Coffman, 1989; Clerici and Shearer, 1994; Seder and Paul, 1994). Studies in transgenic models of tolerance have shown that although Th1 responses resulted in generation of strong autoimmune responses, Th2 responses are associated with the induction of tolerance (Scott et al., 1994). These observations prompted Ochoa's group to determine whether a shift in the T-helper population occurs during tumor progression. These investigators found a progressive loss of Th-1 populations in the spleen of animals that occurred as a result of tumor development. In this model a decreased production of IFN-γ and IL-2 was accompanied by an increased production of IL-4 by T cells from tumor-bearing animals (Ghosh et al., 1995). However, working in a murine mammary tumor model, Lopez's group recently has shown that while tumor progression resulted in decreased IFN-γ production by T cells, in no case was an alteration in the production of IL-2, IL-4 , IL-6, and IL-10 observed (Handel-Fernandez et al., 1997). These results indicated that at least in this model, a shift from Th1 to Th2 was not responsible for the impairment of IFN-γ production observed during tumor progression. In agreement with this finding, the A20 lymphoma model of tolerance described above revealed a profound impairment of IFN-γ production by tumor-antigen specific $CD4^+$ T cells but no associated increase in IL-4 production (manuscript submitted).

The recent finding that the IL-12 receptor β2 subunit may be the cell surface marker that could tag Th1 and Th2 cells (Szabo et al., 1997; Rogge et al., 1997) would provide an extraordinary tool to further resolve the issue of whether clonal diversion represents an operative mechanism of tumor-induced tolerance.

5. Immunosuppression

A large body of evidence has accumulated indicating that progressive tumor growth in animals models as well as in human beings is associated with a state of immunosuppression regardless of tumor location or etiology (Young et al., 1972; Tagasuki et al., 1977; Broder et al., 1978; Paul et al., 1981; Nelson and Nelson, 1987). This is especially true in the setting of advanced disease, which is often accompanied by an state of *global immunosuppression* that affects the different compartments of the immune system. In fact, altered function of T cells (Miescher et al., 1986; Finke et al., 1993; Cardoso et al., 1996), NK cells (Gerson et al., 1981; Lopez et al., 1988; Nakagomi et al., 1993), macrophages (Sotomayor et al., 1991; Bhatia et al., 1995), and dendritic cells (Moser et

al., 1985; Gabrilovich et al., 1996) has been demonstrated in different tumor model systems. Moreover, this profound impairment in the immune system seen in late stage cancer may impose a significant barrier for the successful outcome of immuno-enhancing therapeutic approaches. In fact, studies in experimental models as well as early clinical trials have shown that although these approaches often result in eradication of a small tumor burden, they almost always fail when the tumor is more advanced or has been present for longer periods of time (Pardoll, 1996; Levitsky et al., 1996b). The precise nature of the mechanism(s) leading to this state of global immunosuppression has not been fully elucidated. However, several possibilities have been postulated, including the generation of tumor-induced suppressor T cells (North, 1982; Awwad and North, 1989) and the production of tumor-derived inhibitory factors, such as transforming growth factor-β (TGF-β), IL-10, prostaglandin E2, phosphatidylserine, among others (Mukherji et al., 1995; Huber et al., 1992; Calderon et al., 1994; reviewed by Lopez et al., 1996). In addition, mechanisms such as tumor induction of T cell apoptosis (Hahne et al., 1996) and downregulation of MHC molecules by tumor cells (Doyle et al., 1985) may contribute to the negative outcome observed in late-stage cancer.

Recently, Ochoa and colleagues made an important observation through an analysis of the TCR signaling pathway of T cells obtained from tumor-bearing mice and patients with advanced malignancies. They found that a significant percent of these cells lack a key protein involved in T cell signaling, the ζ chain of the TCR-CD3 complex (Mizoguchi et al., 1992). In addition, expression of the tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ was also reduced. Animal studies revealed that this altered T cell phenotype was not seen early after tumor challenge, but it became evident as the tumor burden increases. Furthermore, the kinetics of these changes correlated with the progression of immunosuppression as ascertained by reduced proliferative responses following antigenic challenge and with reduced cytotoxic effector function *in vitro*. Interestingly, T cells taken from tumor-bearing mice reverted to a normal phenotype when cultured *in vitro* in fresh medium. Despite the reports of similar alterations in T cell signal transduction in different experimental models as well as in human cancers (Finke et al., 1993; Nakagomi et al., 1993), the mechanism(s) behind these changes is not known. However, emerging data point to the potential role of soluble tumor-derived factors as responsible for the alterations in T cell signal transduction seen in the tumor-bearing host. In fact, culture of normal T cells with supernatants from certain tumors resulted in significant alterations in the signaling components of these cells, mimicking the changes observed in T cells from tumor-bearing animals. These *in vitro* findings have been further highlighted by the observations that tumor-infiltrating lymphocytes (TIL) in colorectal and renal carcinomas, as well as T cells obtained from lymph nodes infiltrated with follicular lymphoma, demonstrated an absence of CD3 ζ chain, whereas peripheral blood T cells from the same patients appeared normal (Nakagomi et al., 1993; Schultze et al., 1995). One plausible explanation for these findings could be that at the tumor site, the T cells are exposed to the highest concentration of the putative inhibitory factor(s), resulting in a more profound impairment of their signaling transduction pathway. Recently, Kono and colleagues have shown that hydrogen-peroxide secreted by macrophages isolated from metastatic lymph nodes of patients with melanoma induces down-regulation of CD3 ζ chain in autologous peripheral blood T cells (Kono et al., 1996). Whether these molecule represent the major factor responsible for inducing these changes remains to be elucidated. In any case, it seems that reducing the tumor burden, either by surgical debulking for solid tumors or chemotherapy for hematologic malignancies, appears to lead to the reversal of these alterations and restoration of immune responsiveness. These important observations underscore the necessity of achieving a state of minimal residual disease in order that active immunotherapy strategies may result in a meaningful outcome.

V. BREAKING TOLERANCE

Given the multiple mechanisms that mediate antigen-specific tolerance and limit the magnitude of the antitumor immune response, recent studies have explored the ability to break tolerance and release the brakes that represent a bar-

rier for the induction of effective immune responses against tumor antigens. Of course, one of the major concerns is the potential generation of overt autoimmunity that may ultimately result in severe damage to the host. However, encouraging data have been provided recently indicating that *in vivo* manipulation of CTLA-4—a counterregulatory receptor expressed by activated T lymphocytes that might be involved in the induction of tolerance—may result in the generation of enhanced antitumor responses without triggering deleterious autoimmune responses (Leach et al., 1996).

Interestingly, in the transgenic mouse models developed to examine peripheral tolerance, a different outcome was observed when the γ-interferon gene was expressed under the insulin promoter (Sarvetnick et al., 1990). These transgenic mice developed the abrupt onset of diabetes with progressive destruction of pancreatic islets accompanied by the influx of inflammatory cells. This destruction was mediated by lymphocytes, because a back cross of this transgenic strain onto a SCID background (which do not have mature T or B cells) failed to result in diabetes. The immunity that was generated was long lived and systemic, as these transgenic animals subsequently rejected normal pancreatic islet cells when grafted under the kidney capsule. Similarly, spleen cells taken from these mice were cytolytic for islet cells *in vitro*. These results indicated that the elaboration of an inflammatory cytokine such as γ-interferon does result in the generation and activation of autoreactive T cells. The results do not address, however, whether γ-interferon is in some way directly providing costimulation to islet cell-specific T cells, or rather is leading to the recruitment and activation of antigen-presenting cells into the islets that would then process and present islet cell-specific antigens to T cells for priming.

Interestingly, in different experimental tumor models it has been shown that decreased production of IFN-γ by T cells often occurs during tumor progression. The downregulated production of this inflammatory cytokine has been found during analysis of bulk T cells (Sotomayor et al., 1993; Handel-Fernandez et al., 1997), as well as during the analysis of antigen-specific T cells that were rendered tolerant *early* in the course of tumor progression (manuscript submitted). Whether an association exist between this altered production of IFN-γ and the induction of tolerance *in vivo* is being actively investigated in our laboratory. One interesting question would be whether reversal of this profound impairment of IFN-γ production—by any therapeutic means—would be associated with breaking of the tolerant state, as it was found in the IFN-γ transgenic models of diabetes described previously.

For many years it has been known that infections of diverse etiology may trigger autoimmune responses (Sinha et al., 1990). Recently, Rocken et al. have shown that one mechanism that may account for the observed association between infection and autoimmune diseases is related to the ability of infectious agents to break T-cell tolerance (Rocken et al., 1992). Therefore, the possibility exists that certain infectious agents or their products may be proven to be useful tools in breaking tolerance, especially in the tumor setting, where a full expansion of T cells with reactivity against tumor antigens is needed in order to generate a meaningful antitumor response. In fact, a new generation of cancer vaccines is exploiting viruses and bacterias as tumor antigen vectors with encouraging results. Recombinant viruses and plasmid DNA-encoding tumor-associated antigens have been shown to elicit strong specific antitumor responses capable of inducing regression of established tumors in experimental animal models (Restifo, 1996). Moreover, recombinant *Listeria monocytogenes* cancer vaccine represents the most promising member of the new family of bacterial vaccines (Paterson and Ikonomidis, 1996). In addition to its capacity to secrete tumor antigens that effectively load the MHC class II and class I pathways during the phagolysosomal and cytoplasmic phases of its life cycle, infection with recombinant *Listeria* vaccine results in the production of high levels of IFN-γ, which may be playing a role in breaking the tolerant state that often accompanies tumor progression.

Finally, understanding the mechanisms responsible for the induction and maintenance of tumor-induced tolerance represents a critical task to be undertaken by tumor immunologists in the years to came. The knowledge to be gained in this endeavor will ultimately lead to the generation of more potent immune-therapeutic tools capable of *breaking tolerance* and the ultimate success of cancer immunotherapy.

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